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DR. IVAN MIJAKOVIC (Orcid ID : 0000-0002-8860-6853)

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Importance of protein Ser/Thr/Tyr phosphorylation for bacterial pathogenesis

Julie Bonne Køhler[1], Carsten Jers[1], Mériem Senissar[1], Lei Shi[2], Abderahmane Derouiche[2], Ivan Mijakovic[1,2]*

[1]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kgs. Lyngby, Denmark

[2]Systems and Synthetic Biology Division, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden

*Correspondence:

Prof. Ivan Mijakovic

Systems and Synthetic Biology Division

Chalmers University of Technology

Kemivägen 10

41296 Gothenburg

Sweden

telephone: +46 70 982 84 46

e-mail: ivan.mijakovic@chalmers.se

Abstract

Protein phosphorylation regulates a large variety of biological processes in all living cells. In pathogenic bacteria, the study of serine, threonine and tyrosine (Ser/Thr/Tyr) phosphorylation has shed light on the course of infectious diseases, from adherence to host cells to pathogen virulence, replication and persistence. Mass spectrometry (MS)-based phosphoproteomics has provided global maps of Ser/Thr/Tyr phosphosites in bacterial pathogens. Despite recent developments, a quantitative and dynamic view of phosphorylation events that occur during bacterial pathogenesis is currently lacking. Temporal, spatial and sub-population resolution of phosphorylation data is required to identify key regulatory nodes underlying bacterial pathogenesis. Herein we discuss how technological improvements in sample handling, MS instrumentation, data processing and machine learning should improve bacterial phosphoproteomic datasets and the information extracted from them. Such information is expected to significantly extend the current knowledge of Ser/Thr/Tyr phosphorylation in pathogenic bacteria and should ultimately contribute to the design of novel strategies to combat bacterial infections.

(154 words)

Keywords: Ser/Thr/Tyr Phosphorylation, Pathogenic bacteria, Hanks kinases, BY-kinases, Phosphoproteomics, Host-pathogen interactions, Machine learning, Omics integration, Antibiotic resistance, Antibiotic kinase inhibitors**.**

Abbreviations:

BY-kinase, bacterial tYrosine kinase; CPS, capsular polysaccharide; DDA, data-dependent acquisition; DIA, data-independent acquisition; EHEC, enterohemorrhagic *Escherichia coli*; FHA, forkhead associated; IMAC, immobilized metal affinity chromatography; LMW, low-molecular weight; MRSA, methicillin-resistant *S. aureus*; MS, mass spectrometry; PASTA, Penicillin binding protein And Ser/Thr

kinase Associated; PTM, posttranslational modification; PTP, protein Tyr-specific phosphatase; SH2, Src homology 2; SILAC, stable isotope labeling of amino acids in cell culture; TCS, two-component system; TMT, tandem mass tag; T3SS, type III secretion system; T6SS, type VI secretion system.

Introduction

Protein phosphorylation on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues dynamically regulates the cellular activities in bacteria, archaea and eukarya. By modulating the conformation and macromolecular interactions of proteins, phosphate attachment can change the activity, stability or localization of proteins to guide cellular signaling. Although most thoroughly studied in eukarya, where it is the most common type of posttranslational modification [1], Ser/Thr/Tyr phosphorylation is increasingly recognized for playing wide-ranging and essential roles in the control of bacterial life, including the process of pathogenesis [2-5]. Other phosphorylation systems in bacteria include the phosphorylation of histidines and aspartates (such as in two-component systems (TCSs) and, less commonly, of arginines and cysteines, with considerable crosstalk occurring between the different modification systems [6]. The phosphorylation reaction is catalyzed by distinct types of residue-specific kinases and is reversed by cognate phosphatases to terminate signaling events. Most bacterial Ser/Thr-specific kinases belong to the evolutionary conserved Hanks-type family, which are also present in eukaryotes [7-9]. In addition, bacteria contain a small group of atypical Ser/Thr kinases [10, 11]. Most Tyr-directed kinases in bacteria do not, in contrast to eukarya, belong to the Hanks family, but are bacteria-specific and referred to as BY-kinases (Bacterial tYrosine kinases) [10, 12, 13]. The catalytic domain of BY-kinases is characterized by the presence of Walker A, A' and B ATP/GTP-binding motifs [12, 13]. All BY-kinases, as well as many Hanks kinases, are

transmembrane or membrane-associated proteins that can relay signals from the extracellular environment to the cell interior [9, 12, 13]. Many Hanks kinases contain repeats of so-called PASTA domains (Penicillin binding protein And Ser/Thr kinase Associated) in their extracellular portion that bind cell wall fragments [14, 15]. Table I provides an overview of Ser/Thr- and Tyr-specific kinases identified in a selection of commonly studied pathogenic bacteria mentioned in this review [9, 11, 13, 16-19]. Comparatively, much less is known about bacterial phosphatases, which are also fewer in number. The ones described to date include the phosphoprotein and metal-dependent phosphatases (PPPs and PPMs) acting on Ser/Thr residues, and the conventional and low-molecular weight (LMW) protein Tyr-specific phosphatases (PTPs) [9, 20]. Notably, there are also examples of kinases and phosphatases displaying dual amino acid specificity [5, 21-23]. Detailed descriptions of bacterial kinases and phosphatases and their modes of regulation can be found in other reviews [9, 10, 12, 13, 24].

Bacterial pathogens are defined as any type of bacteria able to cause infection in a host organism. The infection process follows a sequence of events including pathogen host entry, immune evasion, replication, dissemination and persistence. Literature on how bacterial Ser/Thr/Tyr phosphorylation is implicated in each of these steps is accumulating. We therefore start this review by presenting a selection of these functional studies. Current rise in antibiotic drug resistance poses an ever-increasing threat to human health and urgently calls for new therapeutic opportunities. As future therapies may involve targeted inhibition of the pathogenic signaling networks that sustain bacterial survival in the human host, research aiming at obtaining in-depth knowledge of bacterial phosphorylation systems should be prioritized. However, we are still far from having a complete picture of how phosphorylation networks contribute to pathogenesis. Mass spectrometry (MS)-based phosphoproteomics has become a key technology for obtaining global and systematic views of phosphorylation networks in biological systems. This review therefore places particular focus on the use of phosphoproteomics in the study of pathogenic bacteria. We provide a comprehensive overview of phosphoproteomic studies that have recorded phosphorylation sites at a global scale in different types of pathogens and describe the use of phosphoproteomics to understand kinase-

substrate relationships and host-pathogen interactions. Successively, a discussion on current and future technical advancements that should move the field forward with the ultimate goal of obtaining deep and quantitative views of the phosphorylation networks supporting bacterial infections is provided. Furthermore, based on insights obtained from eukaryal phosphorylation, we describe how protein structural and evolutionary information can facilitate qualified predictions of phosphosite functions. We then generally outline how machine learning can help reveal regulatory patterns embedded in complex phosphoproteomics datasets, and how "omics" data integration should allow for a systems level analysis of infectious disease. Finally, we discuss how knowledge of protein phosphorylation can be exploited in the design of new antibacterial therapies.

Control of bacterial pathogenesis by Ser/Thr/Tyr phosphorylation

It is becoming increasingly clear that protein phosphorylation has a potential to regulate most types of cellular processes, and hence it is not surprising that phosphorylation also acts as an important regulator in the context of pathogenesis. To this attest, it has been shown for diverse types of pathogens that inactivation of specific kinases reduces or attenuates their virulence in animal models [14, 25-33]. Kinase-dependent *in vivo* virulence have for example been well-documented for species of *Streptococci* using different infection models. In *Streptococcus agalactiae* (group B streptococci), mutation of the PASTA-domain containing Hanks kinase Stk1 diminishes virulence in a neonatal rat model of sepsis [29], and mutation of the homologous kinase of *Streptococcus pneumoniae*, StkP, decreases the bacterial burden in the lungs and blood of intranasally or intravenously infected mice [30]. Furthermore, in *Streptococcus pyogenes* (group A streptococci) Stk1 has been found essential for disease development in a mouse myositis model of infection [31]. Kinase inactivation has also been seen to reduce the initial colonization of intestinal pathogens [19, 34]. As shown in a hamster infection model, deletion of the Hanks kinase PrkC of *Clostridium difficile* leads to a delay in gut colonization, although it has no significant effect on overall virulence [34]. In some cases, kinase-mediated virulence may depend on the bacterial strain or the infection model. While deletion of the *Staphylococcus aureus* Stk1 Hanks kinase (also known as PknB) in the SH100

strain background causes a significant reduction of virulence in a mouse model of pyelonephritis [32], deletion of Stk1 in the Newman strain has no effect in a septicemia disease model [35]. What is more, a methicillin-resistant *S. aureus* (MRSA) USA300 Δ*stk1* strain was actually seen to cause a more severe infection in a cutaneous mouse model compared to the USA300 wild-type strain [36]. Consistent with the dynamic nature of phosphorylation being important for cell function, phosphatase abrogation also frequently affect *in vivo* infection outcomes [22, 35, 37-39]. This includes the secreted LMW PtpA Tyr phosphatase of *S. aureus*, which enhances bacterial survival within macrophages and promotes mouse liver infectivity [38]. Similarly, the also secretory LMW but dual-specificity phosphatase SP-PTP of *S. pyogenes* is required for normal adherence to human lung cells and *in vivo* virulence in mice. Consistently, SP-PTP overexpression causes hypervirulence [22].

The above-mentioned studies clearly demonstrate the importance of Ser/Thr/Tyr kinases and phosphatases for bacterial virulence. Yet the specific mechanisms by which these enzymes contribute to pathogenicity remain to be systematically elucidated. Individual kinases and phosphatases generally have multiple protein substrates operating in different cellular pathways. Thus, phosphorylation-based regulation is exerted on many levels of pathogen function and must be understood in a context- and species-specific manner. The literature on phosphorylation in the regulation of bacterial physiology and pathogenicity is vast and it is beyond the scope of this review to cover current knowledge in an all-encompassing manner. In this section, we nonetheless seek to illustrate the diversity by which Ser/Thr/Tyr phosphorylation can influence bacterial infections via the control of some critical pathogen functions.

Pathogen cell division and cell wall biosynthesis

Most bacteria harbor a peptidoglycan-based cell wall, which acts to protect the bacterium from lysis in its differing environments. Both cell elongation and the septum formed during cell division require peptidoglycan synthesis, making cell wall growth and division highly inter-linked processes. Protein phosphorylation, especially by membrane-associated Hanks-type kinases with PASTA

repeats, plays significant roles in regulating these processes. For instance, the Hanks kinase StkP is a key regulator of cell division in *S. pneumoniae* and controls the balance between elongated cell growth and septation [40]. In an *stkP* mutant, cell wall synthesis is perturbed resulting in elongated cells [40-42]. During cell division, StkP, together with its cognate phosphatase PhpP, localizes to the septum and regulates a number of cell division proteins [40]. One of these is DivIVA, and DivIVA phosphorylation negatively regulates cell elongation and promotes cell division [41, 43]. StkP can also regulate cell division at the direct level of cell wall peptidoglycan synthesis. At the division site, the peptidoglycan synthase PBP2a forms a complex with the membrane-anchored cofactor MacP. MacP is in turn phosphorylated by StkP, leading to PBP2a activation [44]. A recent study aimed at dissecting the roles of the StkP PASTA repeats during *S. pneumoniae* cell division, showed that while the three membrane-proximal repeats are required for kinase activation and septal cell wall thickness, the membrane-distal repeat ensures proper localization of StkP at the division site and effective cell separation [45]. Interestingly, StkP activation was seen not to depend on PASTA peptidoglycan-binding and thus contrasts the generally accepted view of PASTA-dependent kinase activation [9, 45].

The *S. aureus* Stk1 Hanks kinase is also involved in regulation of cell wall metabolism and division [32]. The cell wall precursor molecule Lipid II has been reported as the activating signal for Stk1, and binding to lipidII variants induces Stk1 autophosphorylation *in vitro* [46]. *In vivo*, Stk1 localizes to the septum following recruitment of the contractile Z-ring forming protein FtsZ. Phosphorylation reduces FtsZ GTPase activity to possibly modulate Z-ring dynamics. In addition, Stk1 phosphorylates the response regulator WalR, which controls the expression of a number of proteins involved in cell wall remodeling [46].

In *Mycobacterium tuberculosis*, we also find a PASTA-domain containing Hanks kinase, PknB, involved in regulating cell wall metabolism. Here, a key PknB substrate is the peptidoglycan synthase CwlM [47, 48]. In its phosphorylated form CwlM interacts with the forkhead associated (FHA)-domain protein FhaA in the cytosol to presumably control peptidoglycan synthesis. Conversely, non-

phosphorylated CwlM localizes to the cell membrane and interacts with the lipid flippase MurJ important for peptidoglycan precursor export (Figure 1A) [48]. Interestingly, in the related, but mostly non-pathogenic *M. smegmatis*, phosphorylation of CwlM quickly diminishes when cells are starved, suggesting that CwlM phosphorylation acts to adjust peptidoglycan synthesis according to nutrient status [47].

Virulence gene expression

Pathogens produce a variety of virulence factors that enable them to colonize host cells and tissues and survive inside their host. Kinase or phosphatase inactivation often significantly alters bacterial gene expression profiles, including expression levels of virulence determinants [31, 36, 37, 39, 49-51]. These phenotypes are due to both direct and indirect effects of kinases and phosphatases on gene transcription. Inactivation of kinases or phosphatases may affect the transcription of genes regulated by direct kinase/phosphatase targets, but potentially also, indirectly, of genes part of the same gene regulatory networks. A common way by which phosphorylation directly regulates virulence gene expression is by modulating the DNA-binding properties of transcriptional regulators [52]. This is the case for the highly conserved catabolite control protein and virulence regulator CcpA in *S. aureus.* CcpA gets phosphorylated on two threonine residues in its DNA-binding domain by Stk1. By abrogating CcpA DNA binding, this phosphorylation event prevents the expression of the critical virulence protein α-hemolysin; a cytotoxin able to induce host cell death [53]. In contrast, Stk1-mediated phosphorylation of another *S. aureus* transcriptional regulator, SpoVG, enhances SpoVG binding to target promoters of virulence genes like *lip* (lipase), *nuc1* (thermonuclease 1) and *capA* (capsular polysaccharide biosynthesis protein) (Figure 1B) [54]. Exemplifying crosstalk between different phosphorylation-dependent signaling systems, *S. aureus* Stk1 is also seen to regulate the TCS response regulator GraR implicated in virulence and antibiotic resistance [55]. GraR phosphorylation increases its DNA-binding activity to proposedly induce expression of genes involved in the modulation of cell-wall charge via wall teichoic acid (WTA) D-alanine incorporation. This is believed to have a global effect on *S. aureus* pathogenicity since WTA charge is important for diverse processes such as cell growth, host infection and attachment, biofilm development and drug

resistance [55]. Similar phosphorylation system crosstalk is seen in *Streptococci* between the CovR/CovS (control of virulence) TCS and the Stk1 Hanks kinase. In *S. agalactiae* and in *S. pyogenes*, the CovR transcriptional regulator can be phosphorylated on Thr65 by Stk1 [39, 56, 57]. CovR acts mainly as a repressor of virulence genes, and phosphorylation of CovR on Asp53 stimulates its DNA binding. On the other hand, a phospho-mimetic CovR-Thr65Glu variant reduces Asp53 phosphorylation and consequently CovR promoter regulation [56, 57]. Consistently, in *S. pyogenes*, the phospho-silenced CovR-Asp53Ala and the phospho-mimetic CovR-Thr65Glu strains were seen to display similar profiles of gene repression to a CovR-deleted strain. Interestingly, compared to Δ*covR*, CovR-Asp53Ala and CovR-Thr65Glu expressing strains were still able to positively influence the expression of some CovR-activated virulence genes, and in accordance caused hypervirulence in a mouse infection model [57]. Similarly, *in vivo* hypervirulence as well as increased blood–brain barrier penetration is seen for S. *agalactiae* CovR-Asp53Ala, CovR-Thr65Glu and CovR-deleted strains [58]. Mechanistic insight into Hanks kinase and TCS crosstalk relevant to bacterial pathogenicity was also recently provided for *S. pneumoniae* [59]. During host invasion, *S. pneumoniae* has to adapt to local fluctuations in pH in both extra- and intracellular compartments. In response to acidic stress, *S. pneumoniae* may undergo acidic stress induced lysis (ASIL), where virulence factors like pneumolysin are released and cause tissue damage [60]. The response regulator ComE controls whether prosurvival or lytic responses are initiated at low pH [59, 61]. More specifically, Piñas *et al*. demonstrated that ComE phosphorylation on Thr128 by the StkP Hanks kinase stimulates ComE DNA-binding, induction of genes involved in H_2O_2 production and cell lysis [59]. In contrast, StkP-deleted and ComE-Thr128Ala mutant strains were blocked in ASIL and displayed increased survival in pneumocytes [59].

Finally, an example of transcription factor regulation via Tyr phosphorylation has been elucidated in enterohemorrhagic *Escherichia coli* (EHEC). Tyr phosphorylation of the sugar-sensing transcription regulator Cra reduces its DNA binding and leads to down-regulation of type III secretion system (T3SS) expression under glycolytic conditions; a condition where T3SS is usually not needed [62]. Recent phosphoproteomic studies have identified multiple transcriptional and post-transcriptional

regulators phosphorylated on tyrosine residues in species like *E. coli* and *Shigella flexneri* [63, 64], and future characterization of these sites should allow us to uncover how Tyr phosphorylation affects virulence gene expression.

Regulation of pathogen exopolysaccharides

Besides governing virulence gene expression, Ser/Thr/Tyr phosphorylation regulates pathogen host invasion and survival through multiple other means. This include the regulation of cell surface capsular and noncapsular exopolysaccharides to facilitate host adherence, biofilm formation, immune system avoidance and antibiotic resistance. Especially BY-kinases are recognized for their role in exopolysaccharide production and export [12, 13]. A well-studied example is the Wzc BY-kinase of *E. coli*, which acts together with its cognate phosphatase Wzb in the synthesis of group 1 capsular and colonic acid polysaccharides [65-67]. The current model proposes that the cycling of Wzc autophosphorylation and Wzb-mediated dephosphorylation drives proper exopolysaccharide assembly [67]. Although the exact mechanism by which this influences exopolysaccharide production is not known, crystallographic studies suggests that Wzc phosphorylation and dephosphorylation cause Wzc to exist in mono- and oligomeric states, respectively [68]. Similarly, the other *E. coli* BY-kinase and phosphatase pair Etk and Etp promotes group 4 capsule formation in enteropathogenic strains [69]. In addition, Wzc and Etk can both phosphorylate the same tyrosine residue on the UDP-glucose dehydrogenase Ugd involved in the synthesis of exopolysaccharide precursors [70, 71]. Whereas Ugd phosphorylation by Wzc regulates colanic acid production, Ugd phosphorylation by Etk is important for resistance to the antibiotic polymyxin [71]. In the context of capsular polysaccharide (CPS) synthesis, crosstalk between Tyr and Ser/Thr phosphorylation has interestingly been seen to coordinate capsular and cell wall synthesis in *S. aureus* by regulating the distribution of precursor molecules common to the two pathways [72]. While the CapA1B1 BY-kinase complex activates CPS synthesis via phosphorylation of the biosynthetic enzymes CapO [73], CapE and CapM, the Ser/Thr kinase Stk1 promotes peptidoglycan synthesis and reduces CPS production by inhibiting CapA1B1 kinase and CapM glycosyltransferase activities [72]. BY-kinases are

also able to coordinate capsule formation with cell division [74]. In *S. pneumoniae*, autophosphorylation of the BY-kinase CpsD, which forms part of a larger CPS assembly complex, is required for proper capsule production at the septum. Furthermore, by modulating the mobility of the chromosome partitioning protein ParB, phosphorylated CpsD acts to coordinate cell constriction with the encapsulation of daughter cells [74].

Host establishment

Upon infection with bacterial pathogens, the host initiates a variety of cellular signaling events that act to stimulate innate and adaptive immune defense mechanisms and ultimately restrict pathogen dissemination. A key virulence strategy utilized by pathogens to subvert these processes and thus promote their own survival is the injection of effector molecules into host cells. Effector translocation is mediated via specialized secretion systems and these are frequently regulated by phosphorylation. Initially, the reciprocal activity of the PpkA-PppA Thr-kinase/phosphatase pair was seen to control secretion from a type VI secretion system (T6SS) in *Pseudomonas aeruginosa* [75]. In the opportunistic pathogen *Vibrio alginolyticus*, T6SS systems are controlled by the PpkA2 Hanks kinase. Besides direct T6SS regulation, PpkA2 controls T6SS expression via a regulatory circuit involving the quorum sensing system [76]. Type III secretion systems (T3SS) are also subjected to diverse modes of Ser/Thr/Tyr phospho-regulation. During infection, enterohemorrhagic *E. coli* uses T3SS both for host attachment and for injection of effector molecules [77]. In addition to transcriptional regulation, as mentioned above [62], EHEC T3SS is regulated by the multicargo chaperone CesT. CesT contains two highly conserved tyrosine phosphorylation sites and these mediate differential effector secretion [78]. In the closely related *S. flexneri*, Tyr-phosphorylation seems to be a negative modulator of virulence and a Tyr-phospho-mimetic mutant of the T3SS-associated ATPase Spa47 displays impaired effector protein discharge [64].

Secreted effector molecules promote *in vivo* survival and propagation by manipulating host signaling and immune defense mechanisms. Many bacterial effectors are actually kinases and

phosphophatases that target host proteins [23, 79-85]. In particular, there are several examples of secreted bacterial kinases and phosphatases disrupting host cytoskeleton structures and immunity [38, 82, 83, 86, 87]. Within the *Yersinia* genus for example, secreted Hanks kinase YpkA (YopO), disrupts cytoskeleton dynamics via phosphorylation of host heterotrimeric Gαq protein [82, 84] and vasodilator-stimulated phosphoprotein (VASP) [85], obstructing effective macrophage phagocytosis (Figure 1C). In *M. tuberculosis*, the secreted Tyr-specific PtkA kinase and PtpA phosphatase, which are encoded in the same operon, are both required for macrophage intracellular growth [83, 88, 89]. Inside macrophages, PtpA dephosphorylates and inactivates the host vacuolar protein sorting-associated protein 33B (VPS33B). This in turn blocks the trafficking of the vacuolar-H^+-ATPase to the mycobacterial phagosome, impeding phagosome acidification, phagosome–lysosome fusion and ultimately bacterial elimination [90]. The molecular mechanism behind PtkA-dependent *M. tuberculosis* macrophage survival is less understood but may occur via PtpA, as PtkA-mediated phosphorylation of PtpA enhances PtpA phosphatase activity [91]. In addition, PtkA is seen to downregulate the macrophage β-galactoside binding protein Galectin 3 involved in regulating apoptosis, and macrophages infected with a Δ*ptkA* strain undergo decreased apoptosis compared to a wild type *M. tuberculosis* strain [89]. The secreted phosphatase PtpA of *S. aureus* is also required for intramacrophage survival, as well as for mouse infectivity, possibly via its interaction with the human cytoskeleton-associated protein coronin-A [38]. *S. pyogenes* encodes two secretory phosphatases, i.e. the Ser/Thr phosphatase SP-STP and the Ser/Thr and Tyr dual-specificity phosphatase SP-PTP, which have both been seen to induce virulence in mouse peritonitis infection models [22, 39]. In addition, both are upregulated upon human pharyngeal cell internalization [92]. SP-STP induces pharyngeal cell apoptosis through both extrinsic and intrinsic host cell stimulation in a manner dependent on its phosphatase activity. In accordance, a SP-STP knock-out strain induces less host cell apoptosis in lungs of infected mice. This suggests that SP-STP interferes with multiple host signaling pathways, and human pharyngeal cells exposed to ectopically expressed SP-STP display altered levels of various pro- and anti-apoptotic proteins as well as deregulated histone phosphorylation [92]. Lastly, to be mentioned, is the interference of bacterial effectors with host innate immune responses via the NF-κB pathway. In *Legionella pneumophila*, the LegK1 Hanks kinase

is secreted via the Dot/Icm type IV secretion system into host macrophages where it activates the NF-κB pathway by functionally mimicking host IκB kinase (IKK) proteins [93].

In some other cases, bacterial pathogens secrete proteins that upon becoming phosphorylated by a host kinase can suppress or hijack host protein function [94-96]. More specifically, Tyr-phosphorylated bacterial effectors can recruit a diverse array of host proteins containing pTyr-binding SH2 (Src homology 2) domains and thereby disrupt diverse host processes [97]. Moreover, bacterial SH2 domains were recently identified in a large group of *Legionella* effector proteins. These were shown to bind Tyr-phosphorylated host proteins with high affinity, proposing a novel mechanism by which bacteria can interfere with host cell signaling [98].

Finally, phosphorylation can guide the metabolic adaptation of pathogens to their changing host environment. Detailed mechanistic insight has been obtained for the regulation of glutamate metabolism by the soluble mycobacterial Hanks kinase PknG [27, 99-101]. Curiously, PknG phosphorylation of GarA induces an intramolecular association in GarA between its phosphorylated N-terminus and its pThr-binding FHA domain, which acts as a switch to relieve the inhibitory interaction with the α-ketoglutarate decarboxylase and NAD^+-specific glutamate dehydrogenase [99, 100]. PknG activation depends on nutrient status [102]. More precisely, periplasm amino acid levels are sensed by the solute binding protein GlnH and the signal is transmitted via the transmembrane protein GlnX to cytosolic PknG [101].

Pathogen persistence

In case of chronic disease, bacteria can employ different mechanisms for persistence of which some are based on protein phosphorylation. Biofilm formation is a well-known strategy for development of persistent infection [103]. Biofilms involve the production of an extracellular matrix that consists of a mixture of proteins, lipids, DNA and different exopolysaccharides. As described above, exopolysaccharide production is regulated by phosphorylation in various ways.

Interestingly, in the dental pathogen *Porphyromonas gingivalis*, the BY-kinase Ptk1 and the metal-dependent tyrosine phosphatase Php1 are required for extracellular polysaccharide synthesis as well as for interspecies community formation with the crucial oral biofilm constituent *Streptococcus gordonii* [104, 105]. Dual-species assemblies are facilitated by the binding of the *P. gingivalis* fimbrial adhesion protein Mfa1 to the *S. gordonii* surface SspA/B proteins, and Ptk1 induces Mfa1 expression by suppressing transcription of the negative gene regulator CdhR [104], possibly via direct CdhR phosphorylation [106]. Ptk1 is itself a substrate for both Php1 and of the LMW tyrosine phosphatase Ltp1, with the latter having a positive effect on CdhR levels and a restrictive impact on *P. gingivalis-S. gordonii* community development [107, 108]. Another important element of persistence is cell dormancy where the stochastic establishment of subpopulations of non-replicating bacteria can confer resistance to antibiotics targeting proliferating cells. Upon drug withdrawal, reservoirs of dormant cells can resume normal growth and re-initiate infections. In *E. coli*, phosphorylation can induce the persistence phenotype via the toxin-antitoxin module HipAB. The toxin HipA is a Ser/Thr kinase that phosphorylates and inactivates the glutamyl-tRNA synthetase GltX. This leads to an increased pool of uncharged $tRNA^{Glu}$, which in turn leads to activation of the stringent response and inhibition of cell growth (Figure 1D) [109]. Dormancy is also a key feature of *M. tuberculosis* infection. After the primary infection, immune-derived granuloma structures can form in which bacteria can reside for decades. Inside the granuloma, *M. tuberculosis* faces a hypoxic and nutrition-limited environment that leads to development of dormant cells. The TCS DosSR constitutes the main regulator of dormancy and is activated in response to hypoxia [110]. Thr-phosphorylation of the response regulator DosR by the PknH kinase enhances DosR DNA binding and thus expression from the dormancy regulon [111]. The *M. tuberculosis* PknG kinase is similarly important for persistence. Indeed, PknG is required for metabolic adaptation to hypoxia and effective survival during latency-like conditions; a role that is mediated via its phosphorylation of the central metabolic regulator GarA [112].

In summary, the above examples demonstrate the importance of studying Ser/Thr/Tyr phosphorylation in order to understand how pathogenic bacteria invade, survive and persist within

their hosts. As shown, bacterial kinases can modify numerous and diverse proteins important for bacterial growth and pathogenicity. The secretion of bacterial effectors that can directly interfere with host phosphorylation signaling furthermore expand the repertoire of phosphorylation-dependent mechanisms driving bacterial infections. Notably, several of the discussed functional studies were inspired from phosphoproteomic datasets, emphasizing how unbiased phosphoproteomic profiling can be used to acquire novel insight into the regulation of bacterial pathogenesis.

Phosphoproteomics in the study of bacterial pathogenesis

Major advancements in mass spectrometry (MS)-based proteomic methodologies over the last two decades have allowed researchers to obtain global and unbiased insight into protein posttranslational modifications at the site-specific level. Consequently, it has revolutionized our knowledge of proteins and their regulation. Nowadays, phosphoproteomics is a mainstream laboratory technique used to identify regulatory events in all kinds of organisms. Mainly Ser/Thr/Tyr-type phosphorylations have been extensively studied in proteomic experiments. This is due to the chemical lability of other types of phospho-bonds, which challenge their detection by typical MS-based protocols. Initially, phosphoproteomic protocols were based on the separation of proteins on 2D-gels where excised phosphorylated proteins were analyzed by MALDI-TOF MS. However, this strategy is biased towards only the most abundant phosphoproteins and can usually not determine the specific modification sites. Thus it was not until the development of gel-free methods coupled to high-accuracy MS that high-throughput and site-specific phosphoproteomic analysis became feasible. The current state and future directions of phosphoproteomics in the study of bacterial pathogens will be discussed below.

Large-scale identification of phosphosites

A typical shutgun phosphoproteomic experiment involves the digestion of total cellular proteins followed by liquid chromatography tandem MS analysis (LC-MS/MS) analysis of sorted

phosphorylated peptides. As phosphorylations are typically sub-stoichiometric and of low abundance, phosphopeptides only represents a tiny fraction of total protein digests [113]. Therefore, their isolation from the rest of the proteome prior to MS analysis is of paramount importance for their successful detection [114, 115]. The most common enrichment methods are immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MAOC; most often with TiO2) [116]. Both methods rely on the affinity of negatively charged phosphate groups for metals. Strong cation exchange chromatography (SCX) can furthermore be used to fractionate peptides prior to phosphoenrichment to reduce sample complexity and thereby improve phosphopeptide MS/MS identifications. Alternatively, phosphopeptides can be enriched for by phospho-specific antibodies [115]. Another important step for maximizing phospopeptide/site coverage is related to the fragmentation patterns of phosphopeptides during MS/MS. Current fragmentation methods have recently been discussed by *Potel et al*. [117]. Phosphoproteomic protocols have primarily been developed for eukarya, for which they are routinely used to identify tens of thousands of sites [118, 119]. Comparatively, bacterial phosphoproteomes are much less explored. Bacterial Ser/Thr/Tyr phosphosites are generally of a much lower abundance compared to eukaryal sites [113, 120, 121], making their identification more challenging. Nevertheless, since the first studies in bacterial site-specific phosphoproteomics [122] , our knowledge of bacterial phosphoproteomes has greatly expanded [2, 114, 123]. Phosphoproteomic studies in various types of bacteria have revealed that phosphorylation affects both basic bacterial physiology, such as central metabolism and gene expression, as well as more pathogen-specific functions [63, 64, 124-136]. A high proportion of studies have been conducted for *M. tuberculosis*, likely reflecting the high prevalence and mortality rates of disease caused by this organism [137, 138]. Together Table II and III provide an overview of the untargeted and gel-free Ser/Thr/Tyr phosphoproteomic studies performed in pathogenic bacteria to date. While initial studies generally identified in the range of 50-500 phosphosites from several mg of starting protein (depending on the species), significant improvements in sample preparation have more recently been reported. For example, it is seen that the removal of interfering bacterial biomolecules, such as phospholipids, peptidoglycans and nucleic acids, prior to phosphopeptide enrichment, can greatly increase phosphosite identifications [139-

142]. *Potel et al.* incorporated benzonase treatment and protein precipitation by methanol/chloroform in their workflow to obtain ultra-pure phosphopeptide samples from which they identified 10 times as many *E. coli* phosphosites compared to a standard protocol [140] . Another method that enhances sample purity is the pre-purification of phosphopeptides by calcium phosphate precipitation (CPP) [143]. CPP treatment significantly expanded phosphosite coverage for the non-pathogenic *Streptomyces coelicolor* [142]. Improved phosphopeptide recovery may also be obtained by using IMAC resins in a column format rather than batch or microtip enrichment [140, 144]. Moreover, species-specific optimization may be required to maximize protocol efficiencies [139].

The recent acquisition of large bacterial phosphoproteomes has allowed the assessment of phosphosite sequence features, representing putative kinase recognition motifs. Phosphorylation consensus sequences have been identified in datasets obtained from *M. tuberculosis* [51, 125], *Helicobacter pylori* [145], *S. flexneri* [64] and *E. coli* [63, 146, 147], including bacterial-specific N- and C-terminal phosphorylation preferences [139, 147]. Interestingly, a phosphoproteomic dataset from the tick-transmitted *Ehrlichia ruminantium* pathogen was enriched in recognition motifs for several eukaryal kinases, suggesting significant host cell interaction [148]. Another general theme of bacterial phosphoproteomes is that they, similar to the eukaryal ones, are dominated by pSer sites, followed by pThr and to a lesser extent, pTyr modifications. One exception is *M. tuberculosis* were pThr sites are more frequently reported [149-151]. However, pTyr sites are likely underrepresented in phosphoproteomic datasets obtained with metal-affinity chromatography as their relative low abundance compared to pSer and pThr sites can result in their disproportional enrichment. Instead, antibody-based pTyr-peptide isolation strategies can greatly improve pTyr identifications [115] with approximately 500 and 1000 pTyr sites identified in *E. coli* and *S. flexneri,* respectively [63, 64]. An alternative strategy may be to use pTyr-binding SH2-peptide domains as a pTyr affinity reagent [152]. As the collection of bacterial phosphoproteomes continues to expand, it will allow more systematic analyses of phosphosite features across different species, potentially revealing interesting differences between pathogens and non-pathogens. To facilitate comparative and

functional studies of bacterial phosphorylation, phosphosite data should be easily retrievable from public databases dedicated to collecting experimentally verified phosphosites. Yet such resources are currently underdeveloped for bacteria compared to eukarya. Curated databases that do include bacterial phosphosites are PHOSIDA, dbPTM and the bacteria-specific dbPSP [153]. These were recently reviewed by *Pagano et al*. [154].

Assaying phosphoproteome dynamics

To obtain more than mere snapshots of cellular phosphoproteomes, phosphoproteomic workflows must be coupled to quantitative methods. Only then can the dynamics of phosphorylation events, and thus their regulatory implications, be understood. Quantification can be obtained either with labeled or non-labeled approaches. Labeling techniques can be either metabolic, such as stable isotope labeling of amino acids in cell culture (SILAC), or chemical; e.g., isobaric tag for relative and absolute quantitation, (iTRAQ), tandem mass tag (TMT) or dimethyl peptide labeling [155, 156]. The disadvantages of using labeling is that only a limited number of labels can be used simultaneously and thus the number of samples that can be compared is restricted. However, several labeled experiments can be combined via a common sample to obtain relative quantification comparing an increased number of samples. For instance, two triple SILAC experiments were combined to record phosphorylation dynamics during *E. coli* culture growth [121]. Importantly, quantitative methods can be used to investigate infection-related phosphorylation. *Verma et al*. for example used a TMT-based quantitative approach to identify phosphorylation events specific to *M. tuberculosis* virulence by comparing virulent and attenuated substrains [125] . Similarly, *Marcelino et al*. used label-free quantification to compare the phosphorylation patterns of virulent versus attenuated variants of *E. ruminantium* that had been grown together with bovine aortic endothelial cells [148]. *Misra et al*. used a hypervirulent strain of *Listeria monocytogenes*, harboring an activating mutation in the virulence gene activator PrfA, to identify virulence-associated phosphorylation events. This approach found the phosphorylation of especially glycolytic enzymes to be upregulated in the hypervirulent strain compared to a non-virulent control; findings that are in line with altered glycolytic activity of *L. monocytogenes* during intracellular growth [157]. Finally, the phosphoproteome of a subspecies of

Francisella tularensis (the causative agent of "rabbit fever") was profiled in response to KCl, which induces T6SS formation required for immune evasion. Together with functional experiments, this study was able to establish Tyr-phosphorylation of a T6SS sheath component to be essential for T6SS biogenesis [158]. Quantitative phosphoproteomics can also shed light on mechanisms of antibacterial drug resistance. For *E. coli*, label-free quantification was used to compare a clinically isolated antibiotic resistant strain with wild type cells grown with or without antibiotics for several time periods. This uncovered numerous cases of phosphorylation events potentially driving antibiotic resistance, such as on transcription factors that regulate resistance [147]. Another study aimed to understand the phenotypic mechanisms that contribute to *M. tuberculosis* tolerance to the first-line antibiotic rifampicin. By examining phosphoproteome dynamics of the Beijing strain B0/W148 in response to high-dose rifampicin, the authors identified upregulated phosphorylation of proteins involved in iron sequestration, which is believed to be linked to antibiotic resistance and dormancy [151].

Assigning substrates to kinases

To fully understand individual phosphorylation events, as well as entire phosphorylation networks, we need to characterize their regulation by kinases and phosphatases. Currently, only very few kinase-substrate pairs have been defined. By analyzing strains with altered levels of kinase activity, quantitative phosphoproteomics can identify phosphorylation events depending on a kinase of interest. Several groups have used this strategy to elucidate the substrates of Ser/Thr kinases in pathogens (see Table III) [76, 159, 160]. For example, to shed light on the mechanisms behind HipA kinase-dependent *E. coli* persistence, HipA and its variant HipA7 were overproduced from ectopic promoters and their substrates sought out by SILAC experiments [146]. Though, the success of such a strategy will depend on the mechanism of kinase activation as kinase overexpression may not by itself upregulate kinase activity if the activating signal is concomitantly low. To identify substrates of the essential *M. tuberculosis* PknB kinase, *Kaur et al*. used a hyperactive mutant version (PknB-GM). This allowed them to identify 73 potential PknB substrates hyperphosphorylated in the PknB-GM background [150]. Another strategy in the search for *M. tuberculosis* PknB substrates was applied by

Turapov et al. They used a conditional deleterious PknB mutant that could successfully grow in a specialized osmoprotective media. Label-free quantitative comparison of PknB-producing and -depleted cells detected 13 potential PknB substrates of which the peptidoglycan amidase homolog CwlM was characterized by further analysis [48]. An alternative to genetic manipulation is the use of chemical kinase inhibitors. A small-molecule inhibitor targeting both PknA and PknB was for example used by *Carette et al.* to uncover *M. tuberculosis* PknA/B substrates by label-free quantification [51]. Notably, among the 68 potential substrates identified in the Carette study, only nine overlap with the *Kaur et al*. dataset, and an overlap of only one and two substrates is seen for each of these studies with *Turapov et al*., respectively. As this is likely to reflect the fact that dissimilar experimental conditions will reveal different substrate sub-pools, it highlights need for extended and multifaceted screening to fully elucidate the substrate repertoire of any given kinase. Even though the above-mentioned studies are clearly invaluable in helping to suggest kinase substrate candidates, precautions must be made in their interpretation. More specifically, one must dissect phosphorylation events directly mediated by an investigated kinase from those mediated by downstream kinases operating within the same signaling cascade. Furthermore, kinases form highly inter-connected networks and perturbation of a single kinase is therefore likely to also influence other signaling nodes [1, 161]. When using chemical inhibitors, one should additionally be aware of unspecific effects occurring due to off-target inhibitor binding. Therefore, complementary cross-validation such as by *in vitro* kinase or co-immunoprecipitation assays is essential to sort out direct from indirect targets.

Host-pathogen interactions at the phosphoproteome level

Knowledge of the molecular interactions that occur between pathogens and their hosts is critical not only for understanding the course of infectious diseases but also for discovering novel targets for future antibacterial therapies. A significant amount of host-pathogen crosstalk involves their posttranslational modifications. In addition to indirect effects exerted by pathogens on host phosphoproteomes via the activation of immune and stress responses, bacteria may, as described earlier, also interfere with host phosphorylation signaling in very direct ways. They may for example

secrete effector kinases and phosphophatases able to target host proteins, or they may interact with host phosphoproteomes via a family of phospho-binding domains present in both host and some bacterial cells [79, 97, 98]. The use of quantitative phosphoproteomics has become instrumental for elucidating pathogen-induced phosphorylation signaling in host cells. For example, the effect of the *Salmonella* pathogenicity island 2 (SPI2) on host phosphorylation was assessed in infected macrophage and epithelial cells by SILAC experiments [162]. SPI2 encodes a T3SS system responsible for secreting a high number of virulent factors, including the *Salmonella* Hanks kinase SteC. Additional quantitative phosphoproteomic profiling of SteC-transfected host cells, in combination with an MS-based SteC binding partner screen, identified the Hsp27 host protein as a direct SteC substrate through which SteC induces actin cytoskeleton remodeling to promote *Salmonella* intracellular growth [162]. Pathogen-induced phosphoproteome alterations have also been investigated for *H. pylori*-infected human gastric epithelial cells [163, 164]. Notably, temporal pTyr-specific phosphoproteomics revealed that changes in host phosphorylation mainly occurred at the early stages of infection compared to a 7 hours post-infection time-point. To further identify host signaling pathways affected by *H. pylori* infection, a wild type strain was compared to mutant strains deleted for components of the *cag* pathogenicity island. This approach allowed dissection of host signaling responses depending on the entire T4SS secretion system and the T4SS effector protein Cag4, respectively. While T4SS induced JNK and p38 kinase activation, Cag4 was required for ERK1 signaling [164]. Using a similar experimental design, time-specific phosphorylation was assessed for human cells infected with either wild-type or mutant enteropathogenic *E. coli* (EPEC) deficient for the virulence-associated T3SS secretion system [165]. This screen revealed activation of host mitogen-activated protein kinase (MAPK) signaling by EPEC infection and identified various T3SS-dependent phosphorylation events associated especially with immune responses, intracellular trafficking and cytoskeletal regulation. Importantly, this study provided novel mechanistic insight into EPEC pathogenesis by identifying T3SS-mediated phosphorylation of the septin-9 protein to be critical for efficient EPEC-host cell adherence [165]. A study on *F. tularensis* also pointed to host MAPK signaling being disturbed upon pathogen interaction. *Fabrik et al*. used SILAC-based quantification to analyze the temporal dynamics of phosphorylation in primary dendritic cells (DC) at

the early stages of infection by either virulent or attenuated *F. tularensis* versions. They found that the DC phosphorylation response underwent distinct phases, which differed between the two strains. Only the virulent strain induced later-phase MAPK signaling, believed to facilitate host survival and immune suppression [166].

Increasing the comprehensiveness of phosphoproteomic datasets

Despite significant recent progress in bacterial phosphoproteomics, as described above, there is still a long way to go before we have fully elucidated the complexity of pathogen phosphorylation networks. Optimizing phosphoproteomic protocols is essential if we are to obtain a deep and global understanding of the phospho-regulatory events that drive infectious diseases. For instance, to identify infection-relevant phosphorylation sites, phosphoproteomic analysis should be performed for bacteria isolated from *in vivo* infections. While experiments performed with free-living cells have shed important light on modification patterns under different growth and virulence conditions, phosphorylation sites should be recorded during live infections to increase the chance of identifying substrates important for pathogenesis. This could include either infected cell lines or mouse models [167]. Temporal changes in pathogen phosphoproteomes should then be systematically recorded over the course of the infection process. Separation of subcellular structures would further allow for a spatial resolution of phosphoproteome dynamics [168]. Moreover, parallel analysis of samples obtained from the infected host would allow one to correlate bacterial and host phosphorylation patterns and obtain integrated insight into host-pathogen interactions. We propose that continued improvements in sample preparation, quantification methods, MS instrumentation and data processing should make such experiments possible. A major challenge is associated with enriching low abundant phosphopeptides from already low quantities of material obtainable from *in vivo* infection models. As bacterial proteins will be highly diluted in the more complex host proteome, the separation of bacteria from host cells must be optimized [169]. Continued developments in phosphopeptide preparation [139, 140, 141] and MS instrumentation [170] will further help to maximize the phosphosite coverage that can be obtained from these samples. Emergence of streamlined and time-effective sample preparation platforms that reduce sample requirements and

optimize sample reproducibility [171] should in particular spur the generation of large-scale bacterial phosphoproteomic studies that require the parallel analysis of a high number of samples. Ideally, workflows should be fully automated to allow rapid and robust high-throughput sample processing. To this end, *Leutert et al.* recently developed an end-to-end automated phosphoproteomic protocol (rapid-robotic phosphoproteomics or R2-P2) where proteins and phosphopeptides are captured on magnetic beads and processed in 96 well plates using a magnetic particle processing robot [172].

The basis for obtaining meaningful biological information from phosphoproteomic datasets is the reproducible quantification of phosphopeptides. Phosphoproteomic quantification methods have traditionally involved labelling due to reproducibility problems of label-free methods where individual sample processing leads to high sample-to-sample variation. Even though SILAC is considered the most accurate quantification method [173] it is not suitable in cases where a high number of samples are to be compared, such as in extended time series experiments, or in cases where metabolic labeling is not feasible. Instead, multiplexing by TMT peptide labeling now allows comparative analysis of up to 11 samples and label-free quantification allows the comparison of an essentially unlimited number of samples. Furthermore, recent innovations in label-free methodologies have greatly justified their usage [174, 175]. A detailed comparison of common phosphoproteomic quantifications methods was recently produced by *Hogrebe et al*. [173]. Their conclusion was that even though label-free quantification and SILAC are the most accurate techniques, MS2-based TMT-based quantification is at present the best method for identifying biologically meaningful regulatory phosphorylation events.

Another source for improving bacterial phosphoproteomics comes from advances in MS acquisition methods. New data-independent acquisition (DIA) setups can boost both the depth and reproducibility of shutgun proteomic analysis [167, 170, 176, 177]. Compared to traditional data-dependent acquisition (DDA), where only the most abundant peptide ions in MS1 are selected for MS2 fragmentation in a semi-stochastic manner, all peptides within a predefined m/z range are co-fragmented and measured together in DIA mode. This way DIA enables systematic measurement of

all peptide ions including very low-intensity/abundance peptides. Better performance in terms of reproducibility and accuracy also makes DIA highly suitable for quantification purposes. Though, due to the high complexity of MS2 spectra resulting from peptide co-fragmentation, DIA holds several analytical challenges and requires sophisticated processing tools. Typical spectral deconvolution methods rely on pre-recorded spectral libraries but more streamlined methods based on *in silico* libraries or neural networks are now on the rise [178-180]. Although DIA has not yet been adapted to bacterial phosphoproteomics, an optimized DIA-based quantitative phosphoproteomic protocol was recently reported for human cells [177]. Using label-free quantification, the authors were able to quantify more than 10.000 phosphorylation sites across hundreds of samples. Other acquisition settings that avoid the random sampling problems of DDA are targeted approaches like selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) [181]. PRM and SRM are highly suitable for experiments that seek sensitive and reproducible analysis of already prespecified phosphopeptides [182]. In summary, the field of phosphoproteomics is still undergoing profound advancements. The constant advent of methodologies improving the sensitivity, robustness and quantification of phosphoproteomic profiling will undoubtedly expand our insight into infection-related signaling from both the host and pathogen perspective. Eventually, optimized protocols will allow us to chart pathogen phosphorylation signatures from a minimum of material, such as from infected patient samples, to suggest biomarkers for precision medicine of the future.

Making sense of phosphoproteomic data

While MS-based studies produce large lists of phosphorylated amino acids, characterizing the function of each of these sites can so far not be performed in a high-throughput manner and thus represent a major bottleneck in phosphoproteomic research. This is for example reflected in the fact that less than 3% of identified human phosphosites have been designated a function [1]. In this section, we will present emerging concepts and methods, primarily arising from the study of eukaryal phosphoproteomes, that can propel the functional interpretation of phosphosites and phosphorylation networks at a global scale. Furthermore, the integration of phosphoproteomics

with other types of omics data should eventually allow us to obtain a more holistic view of infectious diseases.

Functional prioritization of phosphosites

Addressing ways to predict functionally relevant phosphosites from big datasets has become a key point in phosphoproteomics as it allows researchers to prioritize phosphosites for further experimental exploration. Such prioritization is especially important given the suggestion that a noticeable proportion of phosphosites may be non-functional and possibly occur due to random kinase encounters and/or evolutionary neutral drift in regulatory interactions [183-185]. Exhaustive examinations of eukaryal phosphoproteomes have suggested that features such as phosphosite stoichiometry, condition-dependent dynamics, evolutionary conservation, PTM (posttranslational modification) co-localization and structural context can be used to indicate phosphosite functional relevance [1, 185, 186]. For instance, a large fractional stoichiometry is a good indication of phosphosite importance [119, 187]. Though, the opposite is not always true as low-stoichiometry sites might mediate specialized functions for a minor sub-pool of a protein. Methods to systematically determine phosphosite stoichiometry from large-scale studies are becoming increasingly accessible [118, 119, 177].

Phosphosites are generally only slightly more conserved than their non-modified counterparts; a theme seen for both eukarya and bacteria [63, 183, 185, 188, 189]. However, phosphosites with a known or predicted function show higher conservation rates [185, 188]. Moreover, although phosphorylation might not be well conserved at an exact position, it may be conserved when considering a somewhat broader positional window. In fact, conserved so-called phosphorylation hotspots consisting of positionally less-constrained phosphosites can indicate areas of regulatory potential [185, 190]. Eukaryal phosphosites are predominantly found in disordered regions [191] and phosphorylation hotspots situated herein are likely to regulate protein-protein interactions [1, 190]. Hotspot regions are also common in structural domains. By mapping known phosphosites from 40 eukaryal species onto Pfam domains *Strumillo et al*. identified 241 phosphorylation hotspots within

162 diverse domain families [186]. Plotting these regions onto structural data exposed that they were often positioned at protein interfaces or nearby catalytic residues, consistent with phosphorylation regulating protein-protein contact and enzyme activity. In line with the notion that integrating multiple sources of information provides better predictive power compared to single features, computational machine learning methods have recently been developed, as will be discussed later on, to systematically integrate multiple phosphosite attributes in the assessment of phosphosite function.

Because above-mentioned structural and evolutionary patterns of phosphorylation have been extracted from eukaryal phosphoproteomes, they may not necessarily apply to bacteria. Similar comprehensive evolutionary analysis of bacterial phosphosites has so far not been performed due to the relative scarcity in recorded bacterial phosphoprotomes, but should become possible as we accumulate more and better-coverage datasets. In any case, the integration of phosphoproteomic data with structural information should be highly instructive for predicting the specific functional roles for bacterial phosphosites as well. In particular, the visualization of sites in their 3D environment can reveal phosphosites that are physically close to functional protein regions in 3D even though they are far in sequence. By suggesting regulatory potentials of phosphosites not deducible from sequence analysis alone, structural insight can greatly improve hypothesis-driven functional experiments. As manual mapping of phosphosites onto protein structures is a time-consuming process, some online PTM databases have started incorporating PDB (Protein Data Bank) structural information into their user interfaces [192]. A software tool to identify PTMs stored in 3D PDB structures has also been developed [193]. Recently a comprehensive PTM-structural database was launched (PRISMOID accessible through http://prismoid.erc.monash.edu/) in which protein modifications extracted from six different databases have been annotated onto matching PDB structures [194]. Since bacterial phosphosites are still underrepresented in web-based repositories [154], their representation in PRISMOID is also minor. The shortage of adequately solved structures further compromises phosphosite 3D annotations. This notwithstanding, we propose that the next step will be to develop software that can integrate phosphosite-structural information with

phosphoproteomic datasets in an automated manner. By integrating information from different databases and prediction tools, the structural context of individual phosphosites, e.g. disorder, domain and interaction properties, could be extracted at a global scale. Additionally, phosphosites could be automatically mapped onto 3D structures if these are available and visual depictions could be generated. In cases where there is no structural data, it would be highly desirable if, in the future, protein structural prediction tools could alternatively provide some putative structural context [195].

How can phosphoproteomics benefit from machine learning

Machine learning is being increasingly used to make sense of the high-throughput and complex datasets produced in the "big data" era. In short, machine learning is a type of artificial intelligence in which computer systems are set to learn and improve upon their own procedures through training datasets. Deep learning is a next-generation branch of machine learning based on multi-layered neural networks and is best suited for analyzing highly dimensional and structured data available in very large quantities [196]. In the biological sciences, machine-learning methods can be used to identify cellular and molecular patterns embedded in the noise of biological systems [197, 198]. In the field of phosphoproteomics, machine learning has especially been used for identifying contextual features of experimentally validated phosphosites with the aim to be able to predict unknown sites from protein sequences. Numerous different prediction methods have been published, including some that focus on kinase-specific phosphorylation sites [199, 200]. A few prediction tools have been specifically tailored to bacteria [201-203]. The first bacteria-specific phosphosite predictor, NetPhosBac, was developed using neural network algorithms [201]. At that time though, only about 150 bacterial phosphosites, from just two species, were available as a training dataset. The MPSite web resource is a more recent and improved machine-learning predictor trained on more than 3000 unique bacterial pSer and pThr-sites from the dbPSP database [153, 203]. While these and most other methods are solely based on primary, and maybe secondary, structural features, a small predictive performance gain may be obtained by including phosphosite 3D-context information [204]. Importantly, the use of deep learning strategies particularly shows

great promise for improving phosphosite predictions. In contrast to conventional machine learning methods, which involve manual feature selection, deep learning algorithms allow automatic and unbiased discovery of phosphorylation patterns [205, 206]. Owing to their improving performances, *in silico* predictions represent a valuable supplement to experimental phosphosite identification methods. For instance, in addition to being relatively labor- and resource-intense, MS-based phosphoprotemic protocols also pose noteworthy technical limitations. In particular, they allow the identification of only a limited number of sites in any given experiment; i.e. the sites modified to detectable degrees in the specific cellular condition. On top of that, the usage of just a few different amino acid-specific proteases (predominantly trypsin) in sample preparations, results in fractions of phosphopeptides that are either too short or too long for MS detection. This means that a significant portion of sites may persistently go undetected. For these reasons, the continued development of highly reliable phosphosite prediction algorisms could make a crucial contribution to mapping all possible phosphorylation substrates.

Another relevant application of machine learning is in functional prioritization of phosphosites, as it enables integrated evaluation of multiple distinct features that can predict functionality [207]. This was indeed showcased by *Ochoa et al.* who incorporated 59 phosphosite characteristics into a single functional score by training machine learning algorithms on human phosphosites of already known function. Substantiating the applicability of their model, it was able to correctly categorize regulatory phosphosites from a comprehensive reference human phosphoproteome [208].

Finally, it is worth mentioning an example in which machine learning was used to facilitate the challenging task of identifying *bona fide* kinase substrates from *in vivo* studies [209]. As described earlier, quantitative phosphoproteomic screens are often used to elucidate kinase-substrate pairs by comparing kinase-pertubated with unpertubated cells. The interpretation of such datasets is however hampered by the fact that manipulation of a single kinase will alter the phosphorylation of both direct and indirect substrates. To address this issue, *Kanshin et al*. devised a machine learning model that could dissect direct from indirect substrates from a large-scale quantitative

phosphoproteomic experiment involving kinase inhibition. As a list of already known substrates was not available to use as a training set for conventional machine learning, they applied an approach related to positive and unlabeled learning. Interestingly, their model, which was validated for two different yeast kinases, found that direct kinase substrates display higher fold changes and faster dephosphorylation kinetics upon kinase inhibition compared to indirect substrates [209].

"Omics" data integration towards a systems-level understanding of infection

While phosphoproteomics can offer important insight into protein posttranslational regulation, it only offers information on one aspect of a cell's biological state. In concert with massive developments within large-scale molecular biology techniques, such as genomics, transcriptomics, proteomics and metabolomics, there has been an increasing interest in integrating different types of 'omics' data to explore bacterial physiology and pathogenicity in a more systemic manner [210, 211]. Current studies include integration of two or more 'omic' layers. *Deatherage Kaiser et al.* for example used a multi-omics strategy that combined proteomics, metabolomics, glycomics, and metagenomics to elucidate interactions occurring between *Salmonella enterica*, its murine host and the intestinal microbiome, revealing the capability of *S. enterica* to alter gut microbiome composition for its own advantage [212]. *Ramos et al.* integrated genomic, transcriptomic, metabolic and protein structural information to identify candidate targets for the development of novel antibiotics in *Klebsiella pneumoniae* [213]. A few studies have included phosphoproteomic data in their multi-omics approach. A comprehensive integrated multi-omics dataset was prepared for *Mycoplasma pneumoniae*, comprising data from phosphoproteomics, lysine acetylomics, DNA methylomics, transcriptomics, proteomics, protein–protein interactomics, metabolomics and a genome-wide essentiality map [171, 214]. Using this resource to explore the regulatory layers of protein abundance, *Chen et al.* uncovered that antisense non-coding RNAs, lysine acetylation, and protein phosphorylation were all better predictors of protein abundance than mRNA levels [214]. A different study used multi-omics profiling, incorporating quantitative phosphoproteomics, proteomics, transcriptomics, lipidomics and metabolomics, to achieve a deeper understanding of the essential roles played by the PknA and PknB Hanks kinases in *M. tuberculosis* pathogenicity.

Together with chemical PknA/B inhibition, this multisystem analysis uncovered a role for PknA/B in the regulation of cell envelope and protein secretion processes, proposedly via phosphorylation of the essential TCS response regulator MtrA [51]. Similar integration of different omics assays is also being used to elucidate bacterial pathogenesis from the perspective of the host [211, 215]. However, successful integration of heterogeneous multi-omics data under a mathematical model is still facing significant challenges. Differences in data file formats, nomenclatures, dimensionality and quality constitute some of the key obstacles for computational data integration and call for community-wide data standardization [216]. Further improvements in omics data pre-processing, integration and machine-learning analysis are essentially needed to fully capitalize on the huge amount of information embedded in large-scale omics datasets. Looking ahead, multi-omics integration together with machine-learning modelling will likely revolutionize our knowledge on bacterial pathogenesis. In particular, integrating data obtained from both host and bacteria should help to uncover the complex web of molecular interactions that dictates infectious outcomes and ultimately offer a systems-level understanding of infectious diseases.

Phosphorylation in antibacterial therapies

The continuous emergence and world-wide spreading of drug resistant bacterial strains [217] places a high demand on discovery of new antibacterial compounds. Understanding the molecular basis behind both inherent and acquired drug resistance is evidently crucial to devise such novel types of therapeutic strategies. Increased awareness of the essential roles played by bacterial kinases in the control of bacterial growth, pathogenicity and antibiotic resistance has led to their exploration as potential future drug targets. Inspired by drugs already used in the clinic to treat cancer, there is an increasing effort to identify bacterial kinase inhibitors that can obstruct key signaling pathways sustaining bacterial pathogenesis and antimicrobial resistance.

Phosphorylation in antibiotic resistance

Common mechanisms underlying antibiotic resistance include modification or degradation of the antimicrobial agent to render it ineffective, modification of target proteins to reduce drug binding and altered drug influx/efflux. Several studies have found that antibiotic resistance can be driven by bacterial kinases. Especially transmembrane Hanks kinases with PASTA domains are seen to confer resistance to cell-wall inhibiting β-lactam type antibiotics in various Gram positive species [14, 19, 34, 36, 218-221]. Although this may not be surprising given their role in cell division and cell wall homeostasis as described earlier, the mechanism(s) by which Hanks-type PASTA kinases promotes β-lactam resistance are only poorly defined. In *S. pneumoniae* antibiotic sensitive strains, including some clinical isolates, the response to the β-lactam drugs involve the StkP Hanks kinase and its cognate phosphatase PhpP [218, 222]. StkP and PhpP expression as well as StkP phosphorylation is induced upon sublethal exposure to penicillin or cefotaxime. Deleting PhpP augments StkP phosphorylation levels and confer drug resistance, suggesting that StkP inactivation could be a strategy for overcoming *S. pneumoniae* β-lactam resistance [218]. Similarly, *L. monocytogenes* PrkA, *E. faecalis* IreK, *C. difficile* PrkC and methicillin-resistant *S. aureus* (MRSA) Stk1 are all required for resistance to different types of β-lactams [19, 34, 36, 219-221]. In *S. aureus*, β-lactams induce the phosphorylation of the β-lactam antibiotics-sensing protein BlaR1 and this initiates a cascade leading to expression of antibiotic resistance determinants [223]. In *E. faecalis*, β-lactams stimulate IreK autophosphorylation and this in turn leads to phosphorylation of the TCS histidine kinase CroS required for antibiotic resistance [224]. *S. aureus* Stk1 has also been linked to the resistance to vancomycin, which is another type of cell wall-acting antibiotic. More specifically, Stk1 was shown to negatively regulate the DNA binding activity of the vancomycin-resistance-associated response regulator VraR of the VraTSR three-component system [225]. These latter two examples illustrate the sophisticated crosstalk that occur between different phosphorylation systems in the orchestration of drug responses.

Other types of antibiotics work by interfering with DNA replication, such as the quinolones.
In *M. tuberculosis*, phosphorylation of the global transcriptional regulator MgrA by PknB controls its differential induction of the NorA and NorB multi-drug-resistance efflux pumps, correlating with

resistance to different types of quinolone drugs [226]. Other *M. tuberculosis* Hanks kinases implicated in drug resistance are PknF and PknG. In fact, the soluble PknG kinase promotes natural resistance to a broad diversity of antibiotics, including ones that target transcription, translation and cell wall biosynthesis [227], while PknF is believed to reduce the activity of the antitubercular drug ethionamide [228]. Ethionamide is a prodrug that needs to be activated by the monooxygenase EthA and EthA expression is regulated by the transcriptional regulator EthR. Notably, PknF phosphorylation of EthR negatively affects its DNA-binding activity and further studies should determine how this affect *M. tuberculosis* ethionamide resistance [228].

Taken together the above findings emphasize the importance of phosphorylation signaling in bacterial drug responses. Yet it is also clear that substantially more work is needed to fully delineate the diversity of molecular mechanisms behind kinase-dependent drug resistance. Such work will be instrumental for developing new and efficient types of antimicrobials, as discussed below.

Drugging bacterial kinases

Due to their well-documented and multi-faceted roles in pathogenesis and antibiotic resistance, bacterial kinases are currently being investigated as potential new drug targets. While drug discovery studies are also trying to find targets towards histidine kinases [229], these will not be the focus here. Small molecule drugs that target human Ser/Thr and Tyr kinases are already being routinely used in the treatment of several types of cancers and new classes of human kinase inhibitors continue to be experimentally and clinically explored [230]. The majority of these drugs target the ATP binding pocket of the kinase catalytic domain and given their clinical success, it was suggested that similar compounds could be devised against bacterial kinases. Already established small molecule kinase inhibitor libraries have accordingly helped guide bacterial drug studies. Many efforts have especially been made to pharmacologically target the essential PknB kinase of *M. tuberculosis* [231-235]. For example, *Lougheed et al.* first screened a commercial library of more than 50.000 compounds and then optimized on a group of inhibitor candidates by medicinal chemistry [233]. *Chapman et al.* also performed high-throughput screening followed by optimization

of an aminopyrimidine inhibitor [234]. However, while these candidate drugs were very efficient in inhibiting PknB activity *in vitro* (in the nanomolar range), their potency *in vivo* was only modest with minimum inhibitory concentrations (MICs) in the micromolar range. This did not appear to be due to poor cell wall permeability [233, 234].

Another approach to identify kinase inhibitors is by *in silico* modelling. *Wlodarchak et al.* exploited the general structural similarity of bacterial and human Ser/Thr kinases to identify PknB inhibitors by *in silico* screening of a human kinase inhibitor collection [236]. Their *in silico* structural docking revealed a family of imidazopyridine aminofurazans lead compounds with high binding affinity towards PknB. Intriguingly, and consistent with the known role of PknB in cell wall homeostasis, these inhibitors were able to significantly potentiate the effect of β-lactams in several types of mycobacteria and in the related *Nocardia asteroides* [236]. This synergistic concept has also been reported for kinase inhibitors of the non-essential *L. monocytogenes* PrkA and *S. aureus* Stk1 Hanks kinases [219, 223, 237-240]. In *L. monocytogenes*, the broad spectrum kinase inhibitor staurosporine, as well as some more selective repurposed human kinase inhibitors, were seen to inhibit PrkA activity and sensitize cells to β-lactam drugs [219, 237]. Similarly, several studies have identified Stk1-inhibiting molecules from small molecule libraries that sensitize MRSA *S. aureus* strains to β-lactams [223, 238-240]. Notably, Kant *et al.* optimized a quinazoline-based Stk1-inhibiting molecule (Inh2-B1) that potentiates the bactericidal activity of the cephalosporins Ceftriaxone and Cefotaxime on drug-resistant *S. aureus*, both *in vitro* and in an *in vivo* septicemia mouse model with negligible cytotoxic side-effects [240]. Mechanistically, Inh2-B1 was shown to reduce the expression of cell wall hydrolase-encoding genes important for cell wall synthesis and biofilm formation. This effect is likely to occur due to reduced phosphorylation and promoter-binding of the WalR response regulator upon Stk1 inhibition [240]. Thus, in summary, several observations suggest that combined β-lactam and Hanks kinase inhibition therapy could represent a novel strategy to combat clinical β-lactam resistance.

Another line of currently investigated mycobacterial inhibitors are targeted towards the cytosolic PknG kinase [86, 241, 242]. In contrast to PknA and PknB, PknG is not essential but is instead important for host intracellular survival by blocking macrophage phago-lysosomal fusion [86]. By only targeting intracellular mycobacteria, it may be expected that PknG inhibition would pose less selective pressure for the development of resistant strains [243]. Encouragingly, an initial drug screen found that chemical inhibition of PknG at its ATP-binding site allowed effective macrophage clearing of mycobacterial cells via the phago-lysosomal system [86, 241]. Since PknG is also proposed to be crucial for the survival of mycobacterial dormant persisters, antitubercular combination therapies that include PknG inhibition could be a prospective mean to prevent disease reactivation [112, 243]. An extensive overview of presently investigated mycobacterial kinase inhibitors is provided in Khan et al. and Mori et al. [243, 244].

Even though the above-mentioned studies clearly demonstrate the clinical potential of bacterial kinase inhibitors, there is to best of our knowledge so far no reports on Ser/Thr or Tyr kinase inhibitors displaying promising antibacterial activities for single-drug clinical use. Larger scale drug screening and optimization of compound leads is basically needed to make antimicrobial kinase drugs clinically relevant. As for example discussed in Pensinger *et al*. [14], differences in biochemical and antibacterial efficacy of currently tested drugs may be related to limited penetration across the bacterial cell envelope, not allowing drugs to reach their cytoplasmic targets. Another explanation for the discrepancy between *in vitro* and *in vivo* effects could be due to *in vitro* kinase function not properly reflecting kinase activity in the bacterial setting where kinases may be tethered to the membrane and other molecular interaction partners are present. Thus, more knowledge of drug uptake mechanisms and *in vivo* kinase function is needed for optimized drug design. Moreover, to be clinically relevant, new drugs should be highly selective towards only bacterial kinases and not show cross-reactivity with host enzymes. To improve target specificity of an initially identified PknA/B inhibitor, *Wang et al.* produced compound-enzyme co-crystals and identified a pocket in the drug binding site of PknB that was unique to mycobacterial kinases [245]. This allowed them to

modify their compound to achieve a 100-fold selectivity over mammalian kinases. As the structure of for example mycobacterial Hanks kinase domains displays only around 20-30% similarity to eukaryal kinases, there may be several options for designing highly bacteria-specific drugs [243, 246]. Alternatively, inhibitors could be directed towards other kinase regions such as the extracellular PASTA domains, which would circumvent the problem of having to cross the cell envelope [14]. As PASTA domains are highly divergent [247] and not present in eukarya they might be suitable target candidates for drugs with high species specificity. Likewise, BY-kinases could be desirable future drug targets due to their absence in eukarya [248]. In any case, a thorough understanding of kinase structure and function as well as of the molecular basis for inhibitor specificity should be key to guide further development of both effective and species-specific drugs that could reach the clinic.

It should also be mentioned that another currently pursued antibacterial strategy involves the development of inhibitors directed towards secreted bacterial tyrosine phosphatases [249, 250]. In addition, host-directed therapeutics targeting host kinases, represent yet another exciting branch of explored anti-infectious medicines [251]. Host-directed therapeutics may for example inhibit pathogen-hijacked host pathways, or work to boost host immune defenses. Importantly, drugs with such activities can be derived from already commercially approved drugs, a concept known as drug repurposing [252].

Conclusions and Perspectives

While individual phoshoprotein studies have provided valuable information about the function of Ser/Thr/Tyr phosphorylation in specific contexts, a more systematic view of phosphorylation landscapes and their dynamics during different pathogenic stages is needed to fully grasp the phospho-regulatory mechanisms underlying bacterial pathogenesis. This includes the acquisition of spatial and time resolved datasets and possibly, simultaneous analysis of both bacterial and host phosphoproteomes to detect phosphorylation-dependent host-pathogen interactions. In addition,

future developments of single-cell phosphoproteomics will be useful for dissecting phosphorylation networks in heterogenous cell populations. A proposed pipeline for future phosphoproteomic experiments in the study of bacterial pathogenesis is provided in Figure 2. Basically, we need to move from a discovery mode to a quantification mode when it comes to studying bacterial phosphorylation. Quantitative phosphoproteomics techniques are routinely used to study phosphorylation dynamics in eukarya. However, their application in bacterial studies is lagging far behind and bacterial phosphoproteomes remain in general largely understudied. This is partly due to detection difficulties related to lower levels of protein phosphorylation found in bacteria compared to eukarya. Thus, technical optimizations are urgently needed to achieve desired insights into bacterial phosphoproteomes. Data-independent acquisition (DIA) mass spectrometry represents one recent development that may benefit bacterial proteomics as it allows reproducible detection of low abundancy peptides. This strategy has to our knowledge not yet been applied in bacterial phosphoproteomics. Another major bottleneck in understanding the role of phosphorylation in bacterial pathogenesis is the functional characterization of phosphosites generated from MS analysis. To help predict phosphosite function, prediction strategies, relying on evolutionary and structural information of the phosphosite sequence, has been described for eukaryal phosphorylation. As we will gain more information on bacterial phosphorylation, it will be interesting to see whether bacterial phosphosites conform to the same localization patterns. We also argue that mapping of phosphosites onto protein 3D structures, or known domains, should greatly facilitate functional interpretations that can be tested experimentally. We propose that such a contextualization of sites generated from large screens should be automated in the future by the integration of phosphosite datasets with structural databases or predictions. The functional insights deriving from individual phosphorylation datasets could be enhanced by integrating them with other omics data, such as transcriptomics, proteomics, metabolomics and acetylomics, to obtain a systems-level understanding of infectious diseases that can guide future antibacterial therapies. Developments in machine and deep learning algorithms are expected to facilitate information extraction from complex multi-omics data to unravel the contribution of protein phosphorylation to pathogenicity.

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Figures

Figure 1

Schematic illustrations of protein phosphorylation in the control of pathogen life cycle processes such a cell wall biosynthesis (A), virulence gene expression (B), host signaling interference (C) and

host persistence (D). (A) *M. tuberculosis* cell wall synthesis is controlled by the Hanks-type PASTA kinase PknB via phosphorylation of the peptidoglycan synthase CwlM. Left: Non-phosphorylated CwlM is localized at the cell membrane where it interacts with the lipid flippase MurJ, responsible for transporting peptidoglycan precursors across the membrane. Right: An accumulation of uncrosslinked extracellular peptidoglycan is sensed by the PknB PASTA repeats and leads to PknB activation. PknB in turn phosphorylates CwlM as well as MurJ, stimulating their interaction with the forkhead associated (FHA)-domain protein FhaA implicated in peptidoglycan biosynthesis [48]. (B) *S. aureus* Stk1 regulates expression of virulence genes via the phosphorylation of transcriptional regulators. Left: Stk1-mediated phosphorylation of the catabolite control protein CcpA within its DNA-binding domain prevents CcpA binding to its target DNA sequence and expression of the cytotoxic α-hemolysin protein (encoded by the *hla* gene) [53]. Right: Stk1 also phosphorylates the stage V sporulation protein SpoVG, which acts as a transcriptional regulator of virulence genes like *capA , lip* and *nuc1*. SpoVG gets phosphorylated on four N-terminal threonines, and in contrast to the situation for CcpA, SpoVG phosphorylation stimulates DNA promoter binding and hence virulence gene expression [54]. (C) A secreted *Yersinia* Hanks kinase obstructs host cell signaling. Yersinia bacteria secretes the kinase YpkA into host macrophage cells via its T3SS system. Once inside, YpkA is able to phosphorylate host proteins such as the heterotrimeric Gαq protein and the vasodilator-stimulated phosphoprotein (VASP). Gαq is a G-protein coupled receptor subunit and is phosphorylated by YpkA within its highly conserved diphosphate binding domain. This phosphorylation event inhibits Gαq GTP binding and consequently Gαq signaling functions such as actin cytoskeleton regulation [84] . Similarly, YpkA-mediated phosphorylation of VASP prevents VASP-driven actin polymerization. These effects on cytoskeleton dynamics impair effective macrophage phagocytosis [85]. (D) The HipA kinase promotes *E. coli* host persistence via the glutamyl-tRNA synthetase GltX. When HipA is inactive, GltX exists in a non-phosphorylated and active state to promote protein translation. However, GltX phosphorylation by active HipA inhibits GltX catalytic activity, leading to an accumulation of uncharged $tRNA^{Glu}$, ribosome stalling and ultimately, cell growth inhibition [109].

Figure 2

Block chart presenting proposed future pipeline for phosphoprotemics in the study of bacterial infections. 1) Phosphoproteome dynamics should be studied during *in vivo* bacterial infections such as by cell culture or mouse models, and parallel analysis should ideally be performed on bacterial and host samples to facilitate an integrated understanding of host-pathogen signaling crosstalk. Furthermore, samples should be acquired at several time points during the course of infection to obtain time-resolved insight into phosphorylation dynamics. Quantification may be achieved by metabolic protein labeling, chemical peptide labeling or by label-free approaches. 2) Bacterial and host cells should be separated prior to species-specific lysis and protein extraction. In addition, subcellular and/or sub-population fractionation may be performed to obtain spatially-resolved sample analysis. 3) Reproducible and time-efficient analysis of a high number of parallel samples should be possible by automated procedures for phosphopeptide enrichment and clean-up in 96 well formats. 4) High performance LC-MS/MS analysis of purified phosphopeptides together with optimized data acquisition methods, e.g. data-independent acquisition (DIA), should result in datasets of high phosphoproteome coverage and good quantitative reproducibility. 5) Phosphoproteomic data should subsequently be subjected to diverse types of bioinformatic and machine learning analyses to get a systemic view of phosphorylation networks and to generate testable predictions of phosphosite functionality. 6) Finally, integration of phosphoproteomes with other types of omics data obtained from the same infection model should be analyzed by machine learning algorithms to reveal molecular signatures of bacterial infections that could be therapeutically exploited.

Tables

Table 1

Table listing reported Ser/Thr and Tyr kinases identified in a selection of commonly studied pathogenic bacteria. Additional uncharacterized kinases are likely to exist in some of these species. ND, no data.

Table 2

Table listing gel-free and site-specific shutgun phosphoproteomic studies performed in pathogenic bacteria. Quantitative studies are annotated and total numbers of identified phosphosites/phosphoproteins are provided.

Table 3

Table listing phosphoproteomic studies aiming at identifying kinase substrates in pathogenic bacteria. The table provides information on quantitative method used and on numbers of potential kinase substrates as well as total phosphosites/phosphoproteins reported in each study.

Text Boxes

Text Box 1

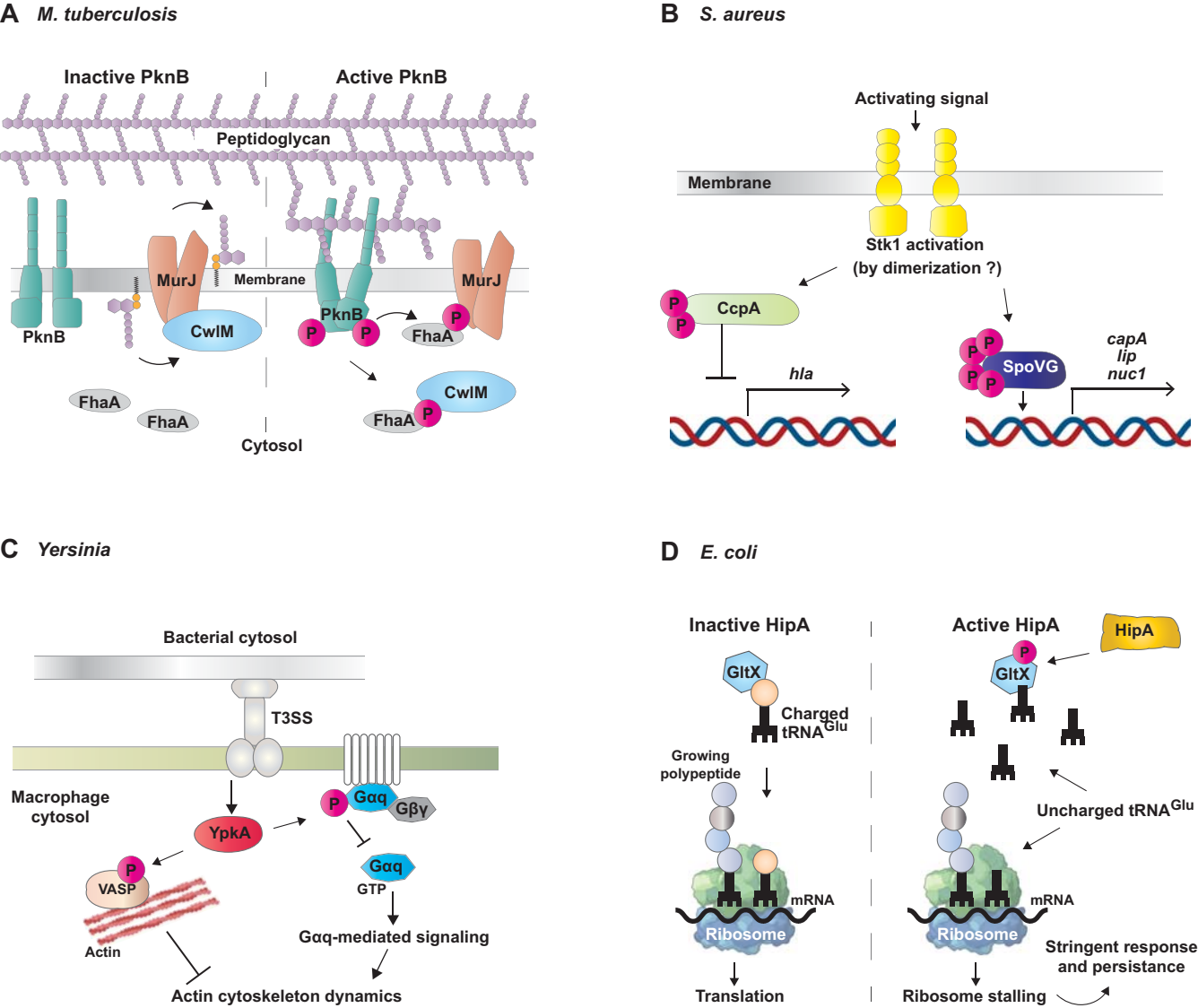
Major take-home messages from this review.

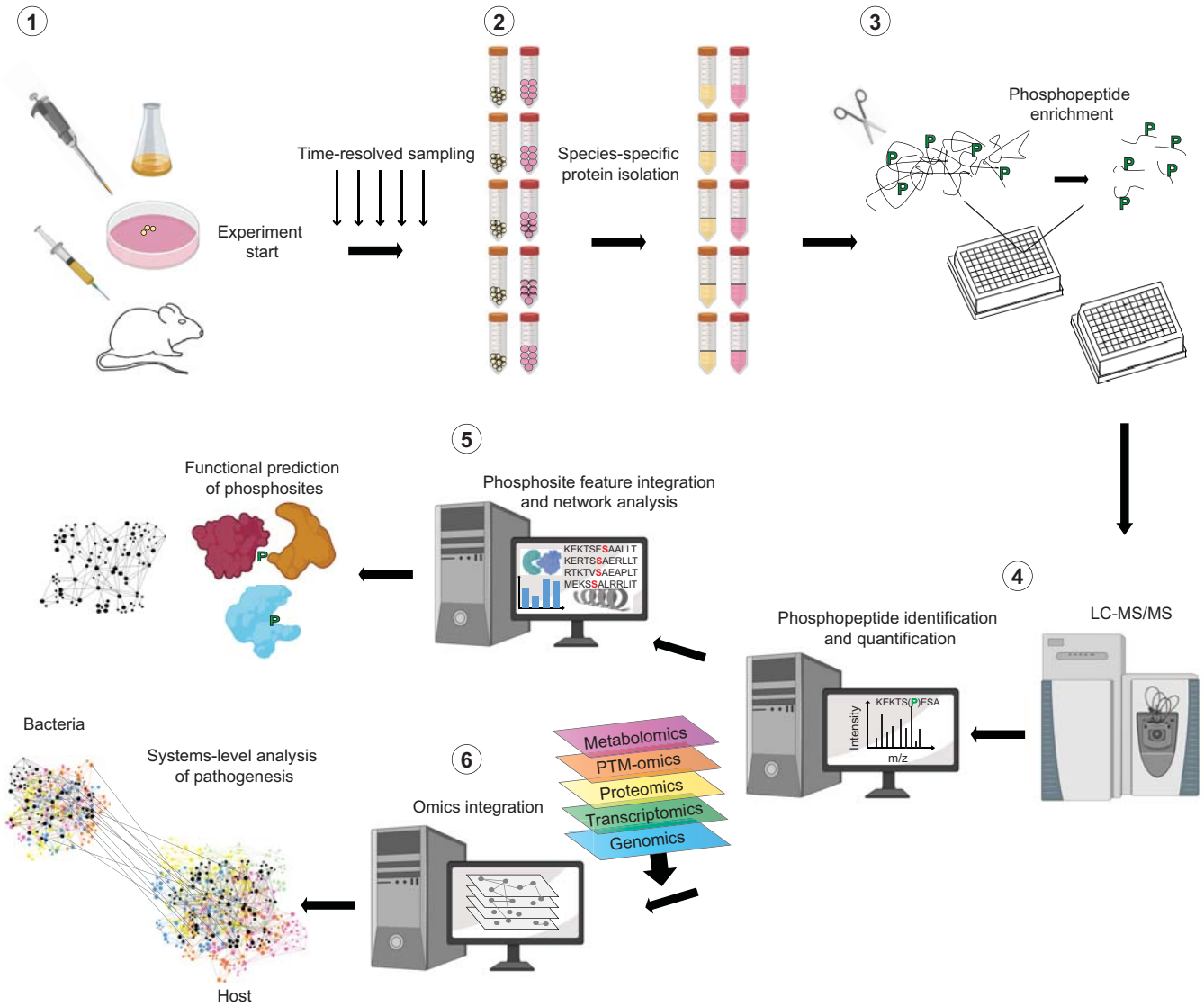
Bacterium	Ser/Thr kinases	Tyr kinases	References
Mycobacterium tuberculosis	PknA; PknB; PknD; PknE; PknF; PknG; PknH; PknI; PknJ; PknK; PknL	PtkA	[9, 13]
Streptococcus pneumoniae	StkP	CpsD; Ubk (only an autokinase?)	[9, 13, 16]
Staphylococcus aureus	Stk1 (PknB)	CapB	[9, 13, 32]
Mycoplasma pneumoniae	PrkC	ND	[9]
Listeria monocytogenes	PrkA	ND	[9]
Escherichia coli	HipA; YeaG; YihE (SrkA); Stk	Etk; Wzc	[8, 9, 11, 13, 17]
Yersinia spp.	YpkA (YopO)	ND	[9]
Pseudomonas aeruginosa	PpkA	WaaP	[9, 13, 26]
Salmonella typhimurium	SteC; RdoA	PutA	[11, 13, 162]
Klebsiella pneumoniae	KpnK	Wzc	[13, 18]
Enterococcus faecalis	IreK (PrkC)	ND	[19]
Clostridium difficile	PrkC; CD2148	ND	[34]

Bacterium	Quantitative	Phosphosites/ phosphoproteins identified	Year	Reference
Acinetobacter baumannii (Reference and invasive multidrug-resistant strains)	No	Strain ATCC17978: 48/41 Strain Abh12O-A2: 80/70	2014	[126]
Acinetobacter baumannii (Clinical Imipenem-resistant and sensitive strains)	No	Strain SK17-S: 410/248 Strain SK17-R: 285/211	2016	[124]
Escherichia coli	No	81/79	2008	[189]
Escherichia coli	Yes (Growth phase analysis)	108	2013	[121]
Escherichia coli	No	766/392	2015	[139]
Escherichia coli	Yes (Antibiotic treatment and resistance)	2509/1133	2018	[147]
Escherichia coli	Yes (Growth phase analysis)	1883 sites	2018	[141]
Escherichia coli	No	>1600 sites	2018	[140]
Escherichia coli	No	512/342 (pTyr only)	2013	[63]
Ehrlichia ruminantium	Yes (Comparing virulent and attenuated strains)	92/58	2019	[148]
Francisella novicida	Yes (KCl treatment)	103 phosphopeptides	2019	[158]
Helicobacter pylori	No	126/67	2011	[127]
Helicobacter pylori	No	Strain OK145: 50 sites Strain TN2GF4: 50 sites	2019	[145]
Klebsiella pneumoniae	No	93/81	2009	[128]
Klebsiella pneumoniae	No	559/286	2015	[139]
Leptospira interrogans	No	27 sites	2010	[129]
Listeria monocytogenes	No	143/112	2011	[130]
Listeria monocytogenes	Yes	242/191	2014	[157]

	(PrfA WT versus PrfA* activated mutant strain)			
Mycobacterium tuberculosis	No	516/301	2010	[131]
Mycobacterium tuberculosis	No	414/214	2015	[132]
Mycobacterium tuberculosis	Yes (Antibiotic treatment)	191/132	2016	[151]
Mycobacterium tuberculosis	Yes (Comparing virulent and attenuated strains)	512/257	2017	[125]
Mycobacterium tuberculosis	No	30/17 (pTyr only)	2014	[21]
Pseudomonas aeruginosa	No	59/28	2014	[133]
Pseudomonas aeruginosa	No	55 sites	2009	[134]
Staphylococcus aureus	No	76/108	2014	[135]
Shigella flexneri	No	905/573 (pTyr only)	2016	[64]
Streptococcus pneumoniae	No	163/84	2009	[136]

Bacterium	Kinase	Quantification method	Potential substrates: sites/proteins	Phosphosites/ phosphoproteins identified	Ref.
Escherichia coli	HipA/HipA7	SILAC	Not easily extractable	1183/632	[146]
Mycobacterium tuberculosis	PknB	TMT peptide labeling	111/73	390 phosphopeptides /258	[150]
Mycobacterium tuberculosis	PknB	Label-free	17/13	ND	[48]
Mycobacterium tuberculosis	PknA/B	Label-free	68/48	1241/470	[51]
Streptococcus agalactiae	Stk1	Not quantitative	10/10	ND	[160]
Vibrio alginolyticus	PpkA2	Not quantitative	10/8	ND	[76]


A M. tuberculosis
Inactive PknB
Active PknB
Peptidoglycan
MurJ
Membrane
MurJ
PknB
CwlM
PknB
P
P
FhaA
P
FhaA
CwlM
FhaA
FhaA
P
Cytosol
B S. aureus
Activating signal
Membrane
Stk1 activation
(by dimerization ?)
P
P
CcpA
hla
P
P
P
P
SpoVG
capA
lip
nuc1
C Yersinia
Bacterial cytosol
T3SS
Macrophage
cytosol
YpkA
P
Gαq
Gβγ
P
VASP
Actin
Gαq
GTP
Gαq-mediated signaling
Actin cytoskeleton dynamics
D E. coli
Inactive HipA
GltX
Charged
tRNAGlu
Growing
polypeptide
mRNA
Ribosome
Translation
Active HipA
HipA
P
GltX
Uncharged tRNAGlu
mRNA
Ribosome
Stringent response
and persistance
Ribosome stalling


1
Experiment start
Time-resolved sampling
2
Species-specific protein isolation
3
Phosphopeptide enrichment
4
LC-MS/MS
Phosphopeptide identification and quantification
KEKTS(P)ESA
Intensity
m/z
5
Phosphosite feature integration and network analysis
KEKTSESAALLT
KERTSSAERLLT
RTKTVSAEAPLT
MEKSSALRRLIT
Functional prediction of phosphosites
6
Omics integration
Metabolomics
PTM-omics
Proteomics
Transcriptomics
Genomics
Systems-level analysis of pathogenesis
Bacteria
Host

Take-home messages

- Ser/Thr/Tyr phosphorylation play wide-ranging roles in bacterial pathogens affecting both basic physiology and pathogen-specific processes.
- Recent advances in bacterial phosphoproteomics has significantly increased our insight into the width of pathogen Ser/Thr/Tyr phosphorylation substrates.
- Further improvements in sample preparation, MS instrumentation, data acquisition and analysis should enable time- and spatially resolved analysis of both bacterial and host cell phosphoproteomes during *in vivo* infections.
- Automated methods to predict the functional implications of bacterial phosphosites should be developed
- Omics integration and machine learning methods will help to obtain a systems-level understanding of the phosphorylation networks driving bacterial pathogenesis.